

# Anchorage-dependent Regulation of the Mitogen-activated Protein Kinase Cascade by Growth Factors Is Supported by a Variety of Integrin $\alpha$ Chains\*

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**Integrin cooperation with growth factor receptors to enable permissive signaling to the mitogen-activated protein (MAP) kinase pathway has important implications for cell proliferation, differentiation, and survival. Here we have sought to determine whether anchorage regulation of the MAP kinase pathway is specific to the  $\alpha$  chain subunit of the integrins employed during adhesion. Human umbilical vein endothelial cells (HUVECs) anchored via endogenous  $\alpha_2$ ,  $\alpha_3$ , or  $\alpha_5$  integrin subunits or NIH3T3 fibroblast cells lines anchored via ectopically expressed human integrin  $\alpha_2$  or  $\alpha_5$  subunits displayed comparable MAP kinase activation upon growth factor stimulation, regardless of the integrin  $\alpha$  chain employed. In contrast, when either cell type was maintained in suspension, growth factor treatment inefficiently activated the MAP kinase pathway. The integrin-mediated enhancement of MAP kinase activation by growth factor correlated with the tyrosine phosphorylation of focal adhesion kinase but was independent of Shc. These data indicate that integrin modulation of the MAP kinase pathway is supported by a variety of integrin complexes and imply that other pathways may be required for the previously reported  $\alpha$  chain-specific effects on cell cycle regulation and cell differentiation.**

The regulation of many cellular events is dependent upon the coordinated effects of cues from adhesive interactions with the extracellular matrix and the presence of circulating growth factors. For example, the anchorage dependence of cell growth has been recognized for many years, and anchorage is also a necessary component of differentiation and survival in many cell types. Recent studies have suggested that adhesion via integrin receptors is able to control growth factor signaling pathways and that this regulation may play a key role in adhesion-dependent cellular responses. Specifically, findings in fibroblasts have indicated that upon growth factor stimulation, cells adherent to the extracellular matrix component fibronectin show enhanced activation of the p42 and p44 forms of MAP<sup>1</sup>

kinase (1–4). Similar observations have been made in endothelial cells, whether cells are stimulated by agonists to receptor tyrosine kinases or G-protein-coupled receptors (5). In addition to this collaborative signaling, engagement of integrin receptors in the absence of growth factor causes a direct transient activation of MAP kinases (6–8).

In collaborative signaling, the point of convergence where integrin signals merge with the growth factor pathway appears to be different depending on the cells and the conditions used. Adhesion-mediated control has been found at the level of receptor tyrosine phosphorylation (2, 9) and at the activation of Raf (1) or MAP kinase/extracellular signal-regulated protein kinase (3). Integrin regulation of the MAP kinase pathway may provide insight into the mechanism of anchorage-dependent effects on cell cycle progression (10). Upon activation, MAP kinases can translocate to the nucleus and regulate the activity of several transcription factors. These events ultimately impinge on the expression of cyclin-dependent kinases and their regulatory subunits, cyclins (11, 12). Anchorage is clearly a necessary component in the regulation of cyclin-dependent kinases, their associated cyclins, and cyclin-dependent kinase inhibitor proteins during the G<sub>1</sub> phase of the cell cycle (13–16).

To date the specific involvement of integrin receptors in adhesion-dependent growth factor signaling to MAP kinases has been addressed either by using function blocking antibodies to the  $\beta_1$  subunit to promote signaling or by showing inefficient signaling when cells are attached by nonspecific interactions to a polylysine-coated surface. However, the integrin subunit specificity of this effect remains unexplored. Several studies have shown important roles for the  $\alpha$  chain of the integrin heterodimer in the regulation of differentiation, growth control, and apoptosis. For example, the decision of myoblasts to follow either a proliferative or differentiation pathway can be controlled by expression of  $\alpha$  chains; exogenous expression of  $\alpha_5$  promotes cell proliferation, whereas expression of  $\alpha_6$  promotes differentiation (17). The proliferative mechanism is transmitted through increases in the cellular levels of the  $\beta_1$  subunit leading to enhanced MAP kinase activity and is also influenced by changes in the levels of tyrosine-phosphorylated focal adhesion proteins (18). In HUVECs and fibroblasts, a role and interesting mechanism for integrin  $\alpha$  subunits in growth control has recently been proposed (19). HUVECs plated on fibronectin or vitronectin entered the S phase of the cell cycle in response to growth factors, whereas a low percentage of cells plated on laminin entered S phase, despite cell spreading (19). Since  $\alpha_5\beta_1$ ,  $\alpha_v\beta_3$ , and  $\alpha_2\beta_1$  are the principal receptors responsible for binding to fibronectin, vitronectin, and laminin, respectively, these data suggest that progression through G<sub>1</sub> is integrin  $\alpha$  chain-specific. These findings correlate with direct signaling via integrins to MAP kinases; the integrins thought to be involved in this process are  $\alpha_5\beta_1$ ,  $\alpha_v\beta_3$ , and

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<sup>1</sup> The abbreviations used are: MAP, mitogen-activated protein; FAK, focal adhesion kinase; EGF, epidermal growth factor; HUVECs, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco's minimal essential medium; PAGE, polyacrylamide gel electrophoresis.

$\alpha_1\beta_1$  but not  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ , or  $\alpha_6\beta_1$  (19). In this model, the transmembrane protein caveolin serves as a link between certain integrins and the Src family kinase, Fyn, which in turn phosphorylates the adaptor molecule, Shc, to initiate the MAP kinase cascade (19, 20). Direct-mediated activation of MAP kinase via caveolin and Shc was found to be independent of FAK tyrosine phosphorylation (19), in agreement with our observation using a different experimental strategy (21). A further example of  $\alpha$  chain specificity in controlling cellular responses is found in studies on extracellular matrix control of mammary epithelial cells. Signals mediated through  $\alpha_6$  but not  $\alpha_2$  integrins collaborate with insulin-dependent signals to suppress apoptosis in these cells (22).

It has been suggested that direct integrin-specific activation of the MAP kinase cascade via caveolin and Shc may contribute to cell cycle progression (19, 20). By contrast, other studies have shown that activation of MAP kinases *per se* is not sufficient to permit cell cycle progression. Thus, constitutive activation of MAP kinase in suspended lung fibroblast cells by expression of an activable form of Raf does not lead to increased expression of cyclin D1 (23). Additionally, endothelial cells partially spread on low concentrations of fibronectin are blocked in the G<sub>1</sub> phase of the cell cycle despite being able to respond to growth factors by activating MAP kinases (24).

Evidently, there are several unresolved issues in the relationship between integrins, MAP kinase, and cell cycle control. We sought to investigate one of these issues, the involvement of specific  $\alpha$  subunits in integrin collaboration with growth factors in the signaling to MAP kinases. Cells were attached via different  $\alpha$  chains and were analyzed for the ability of epidermal growth factor (EGF) to activate MAP kinases. We observed that  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ , and  $\alpha_5\beta_1$  integrins permitted efficient signaling to MAP kinases. These findings point toward the existence of integrin-specific events, other than control of growth factor signaling to MAP kinases, that are important in integrin effects on the cell cycle. Additionally, the ability of cells to respond to growth factor correlated with the tyrosine phosphorylation of FAK but was independent of Shc. Thus, the mechanisms of two forms of integrin-mediated signals, direct and collaborative signaling to MAP kinase, show important differences.

#### EXPERIMENTAL PROCEDURES

**Constructs**—Human  $\alpha_5$  cDNA, subcloned from pECE $\alpha_5$  (25) into the *NotI* and *XbaI* sites of pcDNA3.1 (Invitrogen, Carlsbad, CA), was provided by J.-W. Lee (Department of Pharmacology, University of North Carolina). Human  $\alpha_2$  cDNA in the pSFneo vector was a gift from Dr. M. Hemler (26).

**Antibodies**—Anti-human integrin antibodies, P1E6 and P1D6 (Life Technologies, Inc.) were used to select cells expressing human  $\alpha_2$  and  $\alpha_5$ , respectively. Anti- $\alpha_3$  antibody, P1B6, was used in cell attachment experiments. An anti- $\alpha_2$  cytoplasmic domain polyclonal antibody (27) and a monoclonal anti-human  $\alpha_5$  (Transduction Laboratories, Lexington, KY) were used for Western analysis. Anti-phosphotyrosine clone 4G10 and anti-FAK clone 2A7 were purchased from Upstate Biotechnologies Inc. (Lake Placid, NY) and anti-FAK, clone 77, was from Transduction Laboratories. Shc was immunoprecipitated from cells with a polyclonal antibody (S1630) and visualized by Western blotting with a monoclonal antibody (S14620), both from Transduction Laboratories.

**Cell Selection and Culture**—HUVECs were obtained from Clonetics (San Diego, CA) and maintained according to the supplier's directions. Cells were used between passages 2 and 3. NIH3T3 cells were transfected using SuperFect (Qiagen Inc., Valencia, CA) with vectors expressing human  $\alpha_2$  or human  $\alpha_5$  subunits. Transfected cells were selected by capturing with magnetic beads (DynaL Inc., Lake Success, NY) bound with species-specific antibodies (21). Cells underwent three rounds of antibody-mediated selection. NIH3T3 cells lines were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% bovine calf serum and 500  $\mu$ M G418.

**Flow Cytometry**—Cells ( $5 \times 10^6$ ) were detached using trypsin/EDTA and resuspended in PBS, 0.1% BSA for 45 min on ice, followed by washing in PBS, 0.1% BSA. Secondary antibody incubations using anti-mouse IgG coupled to phycoerythrin (Sigma) were carried out for 45 min on ice. After further washing, cells were fixed in 2% formaldehyde in PBS and analyzed for fluorescence using a Becton Dickinson (Bedford, MA) flow cytometer.

**Preparation of Ligand-coated Dishes or Flasks**—Anti-mouse IgG-precoated MicroCollector flasks (Applied Immune Science, Santa Clara, CA) were incubated with anti-integrin antibodies (P1D6, P1E6, or P1B6 at 2  $\mu$ l/ml) at 4 °C overnight. Tissue culture dishes were incubated with 20  $\mu$ g/ml human fibronectin (Collaborative Biomedical Products, Bedford, MA) at 4 °C overnight. The coated flasks and dishes were blocked with 2% BSA in DMEM for 1 h at room temperature prior to use.

**Cell Adherence and Preparation of Cell Lysate**—For experiments, confluent cells were serum-starved for 4–6 h before detachment by trypsin/EDTA; trypsin activity was subsequently neutralized with 1 mg/ml soybean trypsin inhibitor (Life Technologies, Inc.). Cells were suspended in DMEM with 2% BSA (NIH3T3) or endothelial cell basal medium, 2% BSA (HUVECs) and incubated nonadherently at 37 °C for 45 min in a rotator to allow kinases to become quiescent. Cells were then plated onto antibody- or fibronectin-coated dishes or maintained in suspension and incubated at 37 °C for the indicated times. Following incubations, cells were washed twice with cold PBS and then lysed in a modified RIPA buffer (6). Total cell lysates were cleared by centrifugation at  $16,000 \times g$  for 5 min at 4 °C. Protein concentration in the lysates was determined using the bicinchoninic acid assay (Pierce).

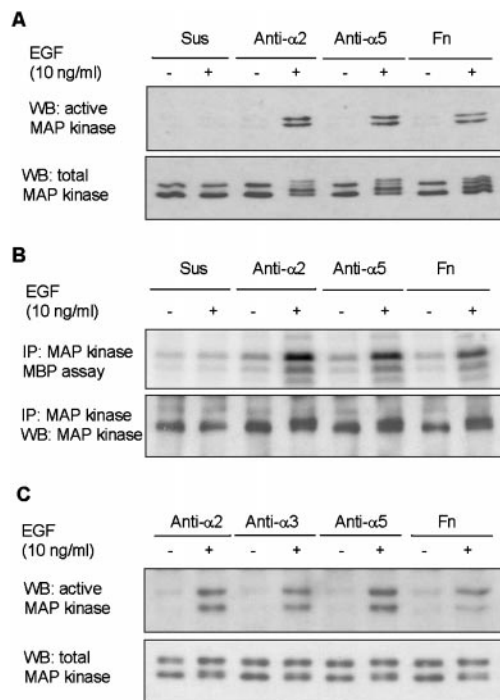
**Immunoprecipitation and Western Blotting**—For immunoprecipitation, cell lysates were first incubated with antibody for 2 h at 4 °C, followed by the addition of protein G-Sepharose and then further incubated for 2 h at 4 °C. Precipitates were washed 3 times with cold RIPA buffer and boiled with SDS-PAGE sample buffer to dissociate the proteins. For analysis by Western blotting, samples were separated by SDS-PAGE under reducing conditions. The proteins were transferred electrophoretically onto polyvinylidene fluoride membranes (Immobilon P, Millipore Corp., Bedford, MA). The membranes were blocked with 1% BSA and 0.1% Tween 20 in PBS overnight at 4 °C and subsequently incubated with primary antibody (1  $\mu$ g/ml) in PBS containing 1% BSA and 0.1% Tween 20 for 1 h at room temperature. Active MAP kinase was detected using an antibody purchased from Promega (Madison, WI), and total levels of MAP kinase were detected using Sc-94 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed in PBS, 0.1% Tween and incubated with goat anti-mouse IgG or goat anti-rabbit IgG peroxidase conjugates (Calbiochem) for 1 h. Immunoreactivity was detected on Hyperfilm using enhanced chemiluminescence (Amersham Pharmacia Biotech). Bands from Western blots were quantified using a GS-670 model densitometer (Bio-Rad).

**In Vitro Kinase Reactions**—p42 MAP kinase was immunoprecipitated for *in vitro* kinase assays using the C-14 antibody (Santa Cruz Biotechnology). Immunoprecipitates were washed three times with cold washing buffer (0.25 M Tris, pH 7.5, 0.1 M NaCl). Immunoprecipitates were resuspended in 40  $\mu$ l of kinase assay buffer containing 10 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10  $\mu$ M ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (370 MBq/ml; NEN Life Science Products), and 10  $\mu$ g of myelin basic protein (Upstate Biotechnology Inc.). Following a 30-min incubation at room temperature, reactions were terminated upon the addition of SDS-PAGE sample buffer and by boiling for 3 min. The samples were subjected to SDS-PAGE, and dried gels were visualized using a Storm 840 PhosphorImager with Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

#### RESULTS

Recent studies have proposed a role for the  $\alpha$  chains of integrin receptors in the ability of cells to activate directly the p42 and p44 MAP kinases, permit cell cycle progression, and avoid apoptosis upon adhesion (19, 20). However, direct integrin-mediated activation of MAP kinases is insufficient for cells to proceed through the cell cycle (19, 29), and the significance of integrin-mitogen collaboration for cell proliferation is still unclear. To explore these issues further, we investigated whether anchorage-modulated signaling to MAP kinases via receptor tyrosine kinases shows integrin  $\alpha$  chain specificity.

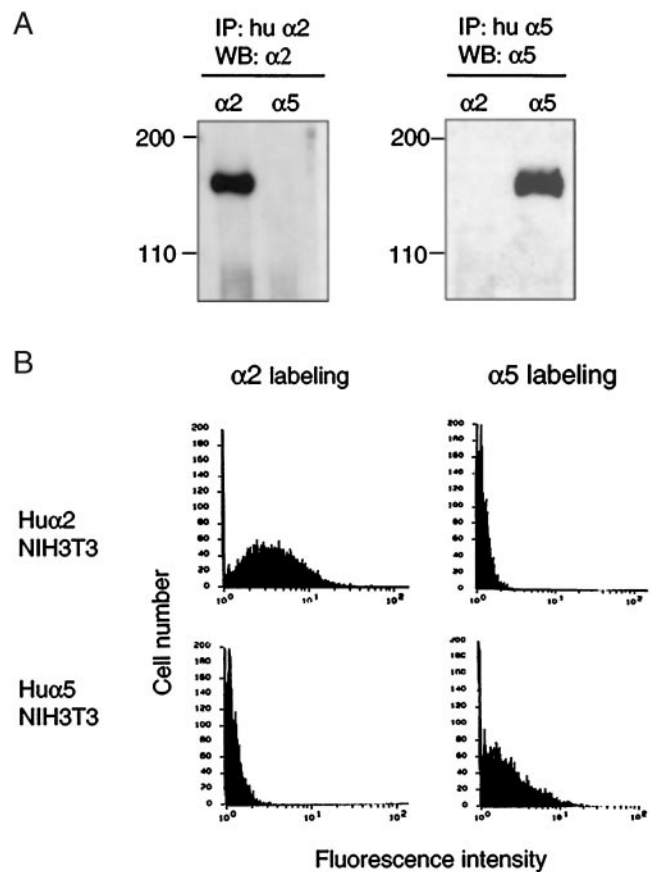
First, we analyzed the effect of cell anchorage via different integrin  $\alpha$  chains in HUVECs. We have previously shown that in HUVECs, EGF signaling to MAP kinases is anchorage-de-



**FIG. 1. Adhesion of HUVECs via different  $\alpha$  integrin subunits supports efficient EGF signaling to MAP kinases.** Serum-starved HUVECs were detached and incubated non-adherently for 45 min at 37 °C. Cells were allowed to adhere to fibronectin (Fn) or antibody ( $\alpha_2$ ,  $\alpha_3$ , or  $\alpha_5$ )-coated plates, or maintained in suspension (Sus) for 2 h in serum-free conditions before treatment with 10 ng/ml EGF for 5 min. Cells were lysed in modified RIPA buffer and analyzed by Western blotting (WB) with antibodies to active MAP kinases and total levels of MAP kinases (A and C) or MAP kinase was immunoprecipitated (IP) and activity measured by an *in vitro* kinase assay using myelin basic protein (MBP) as substrate (B). Results from experiments using  $\alpha_2$  and  $\alpha_5$  antibodies were consistent in four separate experiments.

pendent and that the expression levels of the  $\alpha_2$  and  $\alpha_5$  subunits are very similar (5). To examine the integrin specificity of signaling, serum-starved HUVECs were either maintained in suspension or allowed to adhere to fibronectin, to an anti- $\alpha_5$  antibody (P1D6), or to an anti- $\alpha_2$  antibody (P1E6), before stimulation with EGF. Antibody-mediated attachment of HUVECs was ligand-specific; thus an irrelevant, anti-KT3 epitope tag mouse antibody did not allow attachment. HUVECs attached and spread on the antibody-coated plates almost to the same extent as cells adhering to fibronectin. Western blotting with an antibody that recognizes active forms of p42 and p44 MAP kinases showed that EGF efficiently activated MAP kinases in cells attached via both  $\alpha_2$  and  $\alpha_5$  integrins, but poorly activated MAP kinases in cells maintained in suspension (Fig. 1A). Importantly, the efficiency of signaling on antibody-coated surfaces was similar to that of cells plated on fibronectin (Fig. 1A). These findings were confirmed by the use of *in vitro* MAP kinase assays using myelin basic protein as substrate (Fig. 1B). In addition, cells attached via the  $\alpha_3$  subunit also support efficient EGF signaling to MAP kinases (Fig. 1C). These findings indicate that adhesion-dependent growth factor signaling is not specific to particular  $\alpha$  chains in HUVECs.

To examine further the effects of adhesion via different integrin  $\alpha$  chains on signaling, NIH3T3 cell lines stably expressing either human  $\alpha_2$  (Hu $\alpha_2$ -NIH3T3) or  $\alpha_5$  integrins (Hu $\alpha_5$ -NIH3T3) were established. Expression of these subunits was confirmed by immunoprecipitation studies using human-specific antibodies (Fig. 2A). Flow cytometry analysis demonstrated cell surface expression of the appropriate human integrin  $\alpha$  subunit in the Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 lines;

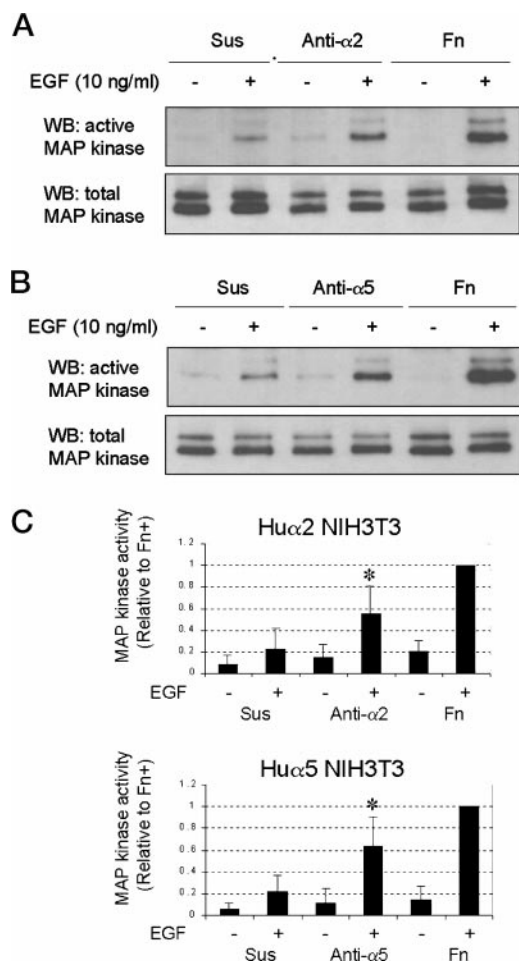


**FIG. 2. Expression of human  $\alpha_2$  and  $\alpha_5$  subunits in NIH3T3 cells.** NIH3T3 cells were transfected with constructs expressing either the human  $\alpha_2$  (Hu $\alpha_2$ ) or human  $\alpha_5$  receptor (Hu $\alpha_5$ ). Cells expressing exogenous integrin subunits were selected and expanded as described under "Experimental Procedures." A, human  $\alpha_2$  and  $\alpha_5$  subunits were immunoprecipitated (IP) from Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 cell lysates with P1E6 or P1D6 antibodies, respectively. Immunoprecipitates were analyzed by Western blotting (WB) with anti- $\alpha_2$  or anti- $\alpha_5$  antibodies. B, Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 were incubated with P1E6 or P1D6 antibodies. Antibody staining was analyzed by incubating with secondary antibody coupled to phycoerythrin and measured on a flow cytometer. The ordinate displays cell number, and the abscissa shows the relative fluorescence intensity on an arbitrary scale.

the expression level in Hu $\alpha_2$ -NIH3T3 was slightly higher than in Hu $\alpha_5$ -NIH3T3 (Fig. 2B). Furthermore, these lines adhered specifically to dishes coated with antibodies that recognized the expressed human integrin subunit and not to plates coated with antibodies to the alternative subunit. These data indicate that the exogenously expressed integrin  $\alpha$  subunits pair with endogenous  $\beta$  subunits and are expressed on the cell surface.

We analyzed integrin-growth factor collaboration upon engagement of different integrins in these NIH3T3 cell lines. Consistent with published findings in wild-type NIH3T3 cells (1, 4), when cells were maintained in suspension or plated on fibronectin and stimulated with EGF, the Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 lines displayed anchorage-dependent signaling to MAP kinases (Figs. 3, A and B). Both the Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 lines adhered rapidly but spread poorly when cells were anchored appropriately via either anti- $\alpha_2$  or anti- $\alpha_5$  antibody. EGF-mediated activation of MAP kinases was equivalently increased in both cell lines plated on the antibody-coated surfaces, above the level of activation in suspension. In both cell lines, the signaling on the antibody-coated surfaces was not as strong as signaling on a fibronectin-coated surfaces, likely representing a lesser degree of cortical actin cytoskeletal structure (4). Levels of active MAP kinase were quantified from four separate experiments and normalized for levels of total

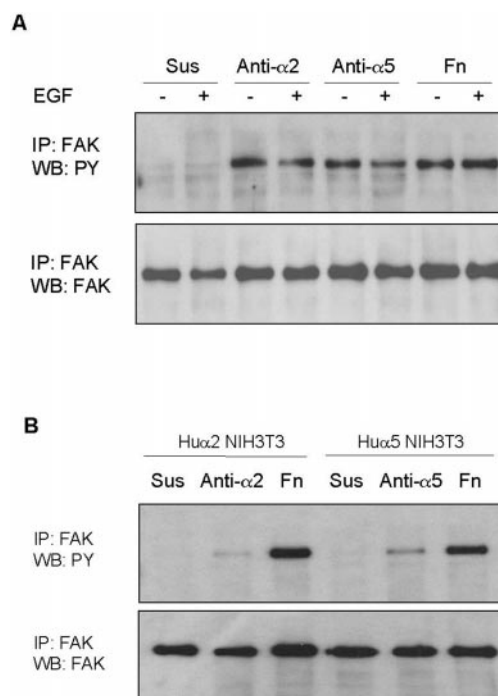




**FIG. 3. Anchorage-dependent growth factor signaling to MAP kinases in NIH3T3 cells is  $\alpha$  subunit-independent.** Serum-starved Hu $\alpha_2$ -NIH3T3 (A) and Hu $\alpha_5$ -NIH3T3 (B) cell lines were detached and incubated non-adherently for 45 min at 37 °C. Cells were allowed to adhere to fibronectin (Fn) or anti-human  $\alpha_2$  or  $\alpha_5$  antibody ( $\alpha_2$  or  $\alpha_5$ )-coated plates or maintained in suspension (Sus) for 2 h in serum-free conditions before treatment with the indicated concentrations of EGF for 5 min. Cells were lysed in modified RIPA buffer and analyzed by Western blotting (WB) with antibodies to active MAP kinases and total levels of MAP kinases. C, bands from Western blots from active and total MAP kinase were quantified using a GS-670 model densitometer. Immunoreactivity for active MAP kinase was normalized for the amount of total MAP kinase for each condition and expressed as a value relative to the value for EGF stimulation in fibronectin adherent cells. Shown is the average and standard deviation from four separate experiments. The enhanced MAP kinase activity in EGF-treated cells adherent to anti- $\alpha$  chain antibodies over cells stimulated in suspension is statistically significant (\* $p$  < 0.05) in both Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 cell lines.

MAP kinase under each condition (Fig. 3C). Hu $\alpha_2$ -NIH3T3 plated on anti- $\alpha_2$  and Hu $\alpha_5$ -NIH3T3 adhering to anti- $\alpha_5$  gave 2.5- and 2.9-fold enhanced activation over cells treated with EGF in suspension, respectively (Fig. 3C). The comparable signaling when cells are attached via either  $\alpha_2\beta_1$  or  $\alpha_5\beta_1$  integrins again indicates a lack of  $\alpha$  chain specificity in adhesion-dependent growth factor signaling.

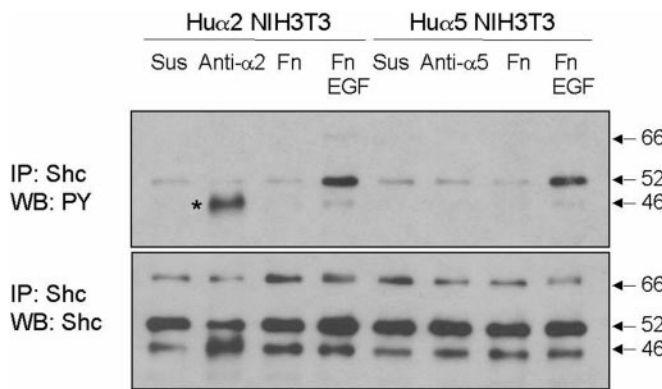
Next we performed experiments designed to provide insight into the mechanistic details of integrin-dependent signaling provided by anchorage to these function-blocking antibodies. We correlated our findings with the effects on two proteins that have been implicated in integrin-mediated signal transduction, FAK and Shc. FAK was immunoprecipitated from HUVECs that were plated on anti- $\alpha_2$ , anti- $\alpha_5$ , or fibronectin for 2 h or maintained in suspension and then in some cases stimulated with EGF. FAK was highly phosphorylated to equivalent levels



**FIG. 4. Anchorage-dependent signaling to MAP kinase correlates with the degree of tyrosine phosphorylation of FAK.** Serum-starved HUVECs (A) or Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 cell lines (B) were allowed to either adhere to anti-human  $\alpha_2$  or  $\alpha_5$  antibody ( $\alpha_2$  or  $\alpha_5$ )-coated plates or fibronectin (Fn) or maintained in suspension (Sus) for 2 h. Cells were lysed in modified RIPA buffer, and FAK was immunoprecipitated (IP) using the C-terminal antibody, clone 2A7. Immunoprecipitated proteins were separated on 8% SDS-PAGE gels and analyzed by Western blotting (WB) with the N-terminal FAK antibody, clone 77. PY, tyrosine phosphorylation.

in HUVECs plated on anti- $\alpha_2$ , anti- $\alpha_5$ , or fibronectin but not in cells maintained in suspension (Fig. 4A). EGF treatment did not alter FAK tyrosine phosphorylation levels. Additionally, FAK was immunoprecipitated from Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 cell lysates that were plated on anti- $\alpha_2$ , anti- $\alpha_5$ , or fibronectin or maintained in suspension. FAK was highly phosphorylated on tyrosine residues when either Hu $\alpha_2$ -NIH3T3 or Hu $\alpha_5$ -NIH3T3 cells adhered to fibronectin but not when cells were maintained in suspension (Fig. 4B). However, the tyrosine phosphorylation of FAK was reduced when cells were plated on antibody-coated surfaces, compared with fibronectin, consistent with the lesser degree of spreading observed. We have previously shown that EGF stimulation of NIH3T3 does not affect the tyrosine phosphorylation state of FAK (21). These observations indicate that the degree of FAK tyrosine phosphorylation correlates with the level of collaboration between integrins and growth factors in the signaling to MAP kinases.

The SH2-PTB domain containing adaptor protein, Shc, was analyzed from Hu $\alpha_2$ -NIH3T3 and Hu $\alpha_5$ -NIH3T3 cell lines under our assay conditions. NIH3T3 cells contain the 46-, 52-, and 66-kDa forms of Shc, although the 52-kDa form is predominant (Fig. 5, lower panel). Shc was weakly phosphorylated on tyrosine residues when immunoprecipitated from cells that were adherent to anti-integrin antibodies or fibronectin, or maintained in suspension in serum-free conditions, in both Hu $\alpha_2$ -NIH3T3 or Hu $\alpha_5$ -NIH3T3 (Fig. 5). However, Shc was robustly phosphorylated on tyrosine residues upon EGF treatment. Further experiments indicated that Shc was highly tyrosine-phosphorylated in response to EGF treatment of Hu $\alpha_2$ -NIH3T3 or Hu $\alpha_5$ -NIH3T3 on anti- $\alpha_2$  and anti- $\alpha_5$ , respectively (data not shown). These findings indicate that Shc does not play a role in the integrin-mediated events that lead to collaborative effects



**FIG. 5. Tyrosine phosphorylation of Shc is controlled by growth factor signaling but not by integrin-mediated anchorage.** Serum-starved HU $\alpha_2$ -NIH3T3 and HU $\alpha_5$ -NIH3T3 cell lines were allowed to either adhere to anti-human  $\alpha_2$  or  $\alpha_5$  antibody ( $\alpha_2$  or  $\alpha_5$ )-coated plates or fibronectin (Fn) or maintained in suspension (Sus) for 2 h. Cells were stimulated appropriately with 10 ng/ml EGF for 5 min before lysis in modified RIPA buffer and Shc was immunoprecipitated (IP). Immunoprecipitated proteins were separated on 10% SDS-PAGE gels and analyzed by Western blotting (WB) with anti-Shc and anti-phosphotyrosine monoclonal antibodies. \*, the 46-kDa immunoreactive band in the anti- $\alpha_2$  lanes represents a nonspecific reaction between the anti- $\alpha_2$  heavy chain and the anti-mouse secondary antibody.

with the MAP kinase cascade but instead is activated solely by growth factor actions.

#### DISCUSSION

We show that engagement of specific integrin  $\alpha$  chains does not determine the ability of fibroblasts or endothelial cells to activate MAP kinase in response to epidermal growth factor treatment. Rather we find that permissive signaling to MAP kinases is more dependent upon the degree of cytoskeletal architecture rather than the specific integrins employed. Thus, tyrosine phosphorylation of FAK, a correlate of the degree of actin organization, serves as a good indicator of the ability of cells to respond to EGF. In our system, it appears that Shc does not play a role in the integrin-mediated events that converge with the growth factor-triggered MAP kinase pathway.

These findings indicate that different mechanisms are employed to activate MAP kinase in collaborative signaling when cells are treated with growth factors after having spread and formed new focal contacts, as compared with signaling directly initiated by adherence. Our analysis of collaborative signaling indicates a role for FAK in this process. FAK has a number of binding partners, such as Src, phosphatidylinositol-3-kinase, and p130<sup>Cas</sup>, that may mediate such an effect. We have yet to determine unequivocally whether FAK plays an active role in this process or is rather simply a marker of the extent of this process. In contrast, MAP kinase can be directly activated by integrins in the absence of FAK tyrosine phosphorylation (19, 22). However, it should be noted that overexpression of FAK is able to enhance direct signaling to MAP kinase (30). A naturally occurring regulator of FAK function, FAK-related non-kinase, is expressed in some cell types (31). FAK-related non-kinase contains the C-terminal focal adhesion targeting sequence of FAK and is thought to compete for FAK-binding sites in focal adhesions (32). Expression of FAK-related non-kinase in our assay had no effect on the growth factor activation of extracellular signal-regulated kinase-MAP kinases (data not shown). This result is not unexpected since FAK-related non-kinase inhibition of FAK function and cell spreading is transient, and the transfected cells are spreading after 2 h of adhesion to fibronectin.

Our data indicate the adaptor protein, Shc, is not involved in the process that makes growth factor signaling more efficient

in integrin-anchored cells. In our system, Shc is not tyrosine-phosphorylated at the 2-h time point when we stimulate the cells with growth factor. It is important to note here that at this time point, direct-mediated adhesion MAP kinase activation has dissipated. Some previous studies have shown that Shc becomes transiently phosphorylated on tyrosine residues upon engagement of  $\alpha_5$ ,  $\alpha_v$ , and  $\alpha_1$  integrin complexes (19, 33). In contrast, others (34, 35) have recently shown that Shc tyrosine phosphorylation levels are unaltered upon fibroblast or smooth muscle cell adhesion to fibronectin under experimental conditions whereby MAP kinase is activated. Our findings do not speak to the issue of direct integrin-mediated phosphorylation of Shc but rather suggest a lack of a role for Shc in the integrin events that collaborate with growth factor signaling to activate MAP kinase.

One common theme between the direct signaling and the co-signaling effects is the importance of the role of the actin cytoskeleton (4, 6, 8). In HUVECs, which spread comparably on antibody-coated surfaces and fibronectin, growth factor signaling to MAP kinases was similar under both conditions. In contrast, NIH3T3 cell lines failed to spread extensively on antibody-coated surfaces and thus exhibited reduced signaling in comparison to fibronectin-adherent cells, although signaling was enhanced over signaling in suspension. Cytochalasin D treatment of cells, causing actin depolymerization, can block both direct and collaborative signaling to MAP kinase (4, 6, 8). Due to the inefficient spreading the human  $\alpha$  chain overexpressing NIH3T3 cell lines on the antibody-coated surfaces, these cells were not particularly suitable for studying direct-mediated signaling to MAP kinase.

The influence that anchorage-dependent regulation of growth factor signaling to MAP kinase exerts on cell cycle components has not been established. However, recent evidence from two separate groups indicates that MAP kinase activity may be necessary, but not sufficient, to permit cell cycle progression (23, 24). Thus, it seems possible that the observed  $\alpha$  chain-specific effects on cell growth (19, 20) probably involve aspects of cell cycle regulation other than activation of the Raf-MAP kinase pathway. Future experiments will be directed to determine the necessary structural regions of integrin receptors for co-signaling to occur and to address directly the importance of efficient activation of MAP kinases to downstream events, such as cell cycle progression. These studies will further elucidate both mechanistic details and the biological importance of adhesion via integrin receptors to critical cellular events.

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